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13. ABSTRACT *(Maximum 200 words)*

Several aspects of breast cancer research, including detection, diagnostics, staging and immunotherapy strategies would benefit from identification of novel tumor antigens important in breast carcinoma. Because cancer patients exhibit both humoral and cellular immunity to tumor associated proteins, patient sera provides a valuable reagent for the identification of potentially immunogenic tumor associated antigens. We have used serological analysis of recombinant cDNA expression libraries (SEREX) for the identification of tumor antigens in a mouse model of adenocarcinoma and in human breast cancer. cDNA expression libraries derived from cell lines or tumor samples have been screened with autologous and allogeneic sera, and reactive clones have been identified. To date we have identified three candidate tumor antigens which may be important in human breast cancer. The clones encoding putative tumor antigens have been purified and are being characterized with regard to nucleic acid sequence, expression patterns, and reactivity with sera from allogeneic cancer patients. The longterm goal of this work is to develop these novel antigens as potential diagnostic and prognostic markers, and as targets for immunotherapy approaches to breast cancer.

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Steven R. Thorp 8/25/98

PI - Signature Date

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Appendix:

Hampton TA, Conry RM, Khazaeli MB, Curiel DT, LoBuglio AF, and Strong, TV. Serological identification of the murine leukemia proviral envelope protein as a tumor antigen in MC38 cells. (Submitted for publication).

INTRODUCTION

The identification and characterization of novel breast tumor associated antigens has potential application to several aspects of breast cancer research. Such antigens may be useful for the detection and diagnosis of breast cancer, as expression may signal the transition from normal to tumorigenic cells. In addition, characterization of tumor associated antigens may contribute to our understanding of the interaction of cancer and the immune system, including the mechanisms by which tumor cells escape immune surveillance. Cancer immunotherapy approaches are likely to be improved with better characterization of the spectrum of proteins capable of inducing immune responses. Finally, tumor associated antigens isolated in this way may be rapidly translated into polynucleotide immunization strategies for the treatment of breast cancer.

Our research is focused on identifying novel breast tumor associated antigens using a serological approach to antigen detection. It has been shown that tumor bearing individuals often develop a limited immune response to their tumor. The production of antibodies directed against tumor antigens has been described for both mutant antigens, such as p53 (1, 2) as well as for nonmutant antigens such as erbB-2 (3) and tyrosinase (4). We proposed to use patient sera as a reagent to identify tumor antigens. This approach has been termed SEREX (4-6), for serological identification by recombinant expression cloning. Our specific aims are as follows:

1. Identify candidate tumor associated antigens by screening breast tumor-derived cDNA libraries with autologous/allogeneic patient sera.

2. Characterize putative tumor antigens at the molecular level.

We first used this strategy in a mouse model of adenocarcinoma to validate the approach in our hands. For these studies we immunized C57/BL6 mice with the syngeneic colon adenocarcinoma cell line, MC38. The preliminary data of our original proposal describes the experimental design, in which serum from immunized animals was used to identify tumor antigens from MC38-derived cDNA libraries. Our work in the first year of the current grant has included more complete characterization of these putative tumor antigens. As stated in our work statement for this period, we have also constructed and screened cDNA libraries from breast cancer tumors and cell lines. In addition, we have begun characterizing putative breast tumor antigens identified in this manner.

Statement of Work:
Aim #1:
1. Obtain breast tumor tissue and matched serum. Construct cDNA libraries and screen with autologous and allogeneic serum (months 1-18)

Aim #2:
1. Plaque purify positive clones, sequence, identify mutations (months 6-24)
2. Analyze expression pattern (months 10-30)
3. Screen positive clones with additional cancer patient sera (breast and other) for reactivity (months 18-36)
4. Evaluate clones for induction of immune response in mice (months 24-36)

BODY
METHODS

Acquisition of tumor and serum samples:
Mouse model of adenocarcinoma: The cell lines and immune sera for identification of tumor antigens in a mouse model were described in our original proposal.

Human breast cancer tumor antigen identification: We acquired a cDNA library derived from four different breast cancer cell lines (MDA-MB-453, MCF-7, T47D, and ZR75-1) from a colleague at UAB, J. M. Ruppert, M.D., Ph.D. We acquired breast tumor samples through the UAB tissue Procurement Facility. We also acquired twenty normal human serum samples from existing samples at UAB's Cancer Center. Serum samples from breast cancer patients were acquired through the Tissue Procurement Facility as well as from existing samples at the Cancer Center. All samples were acquired with IRB approval.

Construction and Screeing of cDNA libraries

For construction of cDNA libraries, total RNA was isolated using RNA Stat 60 RNA isolation reagent (Tel-Test B) according to the manufacturer's directions. Briefly, an appropriate amount of frozen tumor (100 - 500 mg) was pulverized using a mortar and pestle and rapidly immersed in RNA-Stat-60 reagent. After homogenization and a 5 minute spin at 2,000 rpm, the sample was processed according to the manufacturer's directions. The quality and quantity of the total RNA was assessed by denaturing agarose gel electrophoresis. mRNA was isolated by passing the total RNA twice over an oligo-dT column (PolyA-Quik mRNA Isolation Kit, Stratagene). Five to seven micrograms of mRNA was used to construct a cDNA library in the Zap Express vector (Stratagene), according to the manufacturer's directions, with minor modifications. Briefly, mRNA was reverse transcribed using an oligo dT primer with an internal *Xho*I site as well as a random primers which included a *Xho*I restriction site at the end (random primer XXXCTCGAGXXXXXX). The reverse transcription reaction included 5-methyl dCTP. *Eco*RI adapters, were added by ligation and the cDNA restricted with *Xho*I. The cDNA was passed through a size exclusion column (Pharmacia) which eliminates cDNAs smaller than 400 bp in size. cDNA fragments were cloned into the λZapExpress vector, packaged according to the manufacturer's instructions, and used to infect *E.coli* cells. As a preliminary characterization of the library, inserts from twenty-five randomly selected recombinant plaques were amplified by the polymerase chain reaction (PCR) using T3 and T7 primers to determine insert sizes range. This analysis revealed insert sizes ranging from approximately 350 bp to 3.5 kb. Greater than 95% of plaques were recombinants as determined by blue/white screening.

Screening of cDNA Libraries: For immunoscreening, recombinant plaques were plated at a density of approximately 25,000 plaques per 150 mm plate, and 20 plates were screened at a time. Although this is slightly less dense than recommended in some protocols (50,000 per plate is often suggested), we have found this to be a more manageable number for identification of reactive plaques. After a four hour incubation at 42°C, protein expression was induced by incubation of the plates with nitrocellulose filters saturated with isopropyl β-D-thiogalactoside (IPTG) for an additional four hours. Filters were blocked with 1% BSA in Tris-buffered saline (TBS; 20 mM Tris, pH 7.5, 150 mM NaCl) and screened with patient sera. Primary sera was preabsorped with *E. coli* phage lysate (Stratagene) and diluted 1:250 for screening. After incubating filters with diluted sera, the filters were washed with TBST (TBS with 0.05% Tween 20 [Sigma]) and incubated with alkaline phosphate-conjugated goat anti-human IgG (H+L) antibodies (Jackson Labs) at a dilution of 1:5,000 for one hour at room temperature. After washing, an NBT/BCIP colorimetric assay (Vector Labs) were used to identify positive clones. Positive plaques were picked and purified to clonality for further study.

Screening for IgG cDNA. As has been described (5), the majority of reactive clones identified in a screen actually represent expressed portions of Ig molecules, presumably derived from blood and infiltrating lymphocytes. These false positives are rapidly identified during plaque purification by testing the plaque for reactivity with secondary antibody alone. Plaques which are reactive with secondary antibody alone in this assay are discarded.

Isotype analysis. Upon plaque purification, the isotype of the reactive antibodies are determined using a human isotype specific antibodies (Southern Biotech), according to the recommended procedure.

Antigen Characterization

DNA sequencing. DNA was sequenced by thermal cycle sequencing (ThermoSequenase, Amersham). Approximately 200 ng of template DNA will be included in a reaction of reaction buffer, primer, Thermosequenase polymerase. The reaction was divided into the four termination mixes (α-^{33}P labeled ddNTPs), and subjected to thermocycling for 35 cycles. After the addition of stop solution, the samples were electrophoresed on a 6%, glycerol tolerant, denaturing gel. In some instances, we have used the DNA sequencing core facility at UAB for high quality automated sequencing

Southern blot analysis: Genomic DNA was isolated by incubation in 50 mM Tris, pH 8.0, 10 mM NaCl, 10 mM EDTA, 0.5% SDS with 100 µg/ml Proteinase K overnight at 55°C. DNA was extracted with phenol, then chloroform, then precipitated with ethanol and resuspended in TE (10 mM Tris, pH 8.0, 1 mM EDTA). Approximately 10 µg of genomic DNA was digested with the indicated restriction enzyme and electrophoresed on a 1% agarose gel. After treatment with 0.25N HCl, denaturation (0.4M NaOH, 1.5M NaCl) and neutralization (1M Tris, pH 7.5 1.5M NaCl), the gel was transferred to nylon membrane in 20x SSC. Approximately 75ng of cDNA insert from the plaque of interest was labeled with α-^{32}P-dCTP by random prime labeling (Pharmacia) and hybridized to the membrane overnight at 65°C in a solution of 6xSSC, 10x Denhardt's, 100µg/ml sheared salmon sperm DNA, 10% dextran sulfate and 1% SDS. After hybridization, the blot was washed successively in 2xSSC with 0.5% SDS, 1x SSC with 0.1% SDS, and 0.1% SSC with 0.1% SDS; and autoradiographed at -70°C for a period of 1 to 4 days.

Northern blot analysis: Eight micrograms of total RNA was electrophoresed on a 1.2% denaturing agarose gel, transferred to a nylon membrane (Hybond, Amersham) in a solution of 10x SSC. The membrane was hybridized with a ^{32}P-radiolabeled probe overnight at 65°C in a solution consisting of 6xSSC, 5x Denhardt's, 100µg/ml sheared salmon sperm DNA and 0.5% SDS. The blots were washed as described above. To control for loading differences, the blot was stripped and rehybridized with a probe specific for the β-actin gene.

Protein Production Recombinant protein was produced by a phage lysis method. Briefly, positive phage were incubated in liquid cultures with *E. coli* until lysis occurred. Cell debris was removed by centrifugation and the supernatant containing the protein was concentrated approximately 10-fold by centrifugation using a centricon concentrator with a 30 kDa MW cutoff. The quality and quantity of the lysate preparation was determined by SDS-PAGE and Coomassie blue staining.

Western blot analysis *E coli* phage lysates were resolved on an 8% SDS-PAGE gel according to standard methods. Proteins were transferred by electroblotting to a PVDF membrane and blocked in TBST with 1% BSA. Antibody incubation, washes and colorimetric development was performed as described above (screening of cDNA libraries).

RESULTS

A. Characterization of tumor antigens in a mouse model of adenocarcinoma.

We have largely completed studies of tumor antigen identification in a mouse model of adenocarcinoma. Using the SEREX approach, we identified the envelope protein of the murine endogenous leukemia virus as a tumor antigen in MC38 colon adenocarcinoma cells (see enclosed manuscript, submitted). This protein encoded by the 1.4 kb positive clone described in the preliminary data of our original proposal. By Northern blot analysis, expression of the envelope sequence was not detected in normal colonic epithelium, but it is expressed in a variety of murine tumor cell lines, including both spontaneously arising (B16, 4T1, and 4T07) and carcinogen-induced tumors (MC38, EL-4, P815, and

CT26). Expression is seen in tumors arising from different tissues, including those derived from mammary carcinoma (4T1 and 4T07), and from different strains of mice. These features suggest the env protein may act as a shared tumor antigen. Southern blot analysis revealed the presence of novel bands in the MC38 DNA compared to genomic DNA from a C57BL/6 mouse. This suggests that the *env* sequences have undergone amplification and reintegration into the MC38 genome (see appendix). To determine whether the envelope protein is immunogenic due to the presence of a mutation, we next sequenced the full length envelope gene expressed in the MC38 cells and compared it to the prototypical env sequence (AKV-type, 7), and to the normal sequence determined from genomic DNA of a C57BL/6 mouse. No mutations were identified and thus this protein represents a nonmutant tumor antigen which presumably is immunogenic based on its expression in the tumor cells. Finally, we have begun to examine the ability of this protein to function as a tumor rejection antigen. Mice were immunized with the 1.3 kb clone encoding the carboxy one-third of the envelope sequence, the clone we first isolated. Immunization against this self antigen resulted in strong production of anti-envelope antibodies (of subclasses IgG1, IgG2a, and IgG2b, with animal to animal variation).

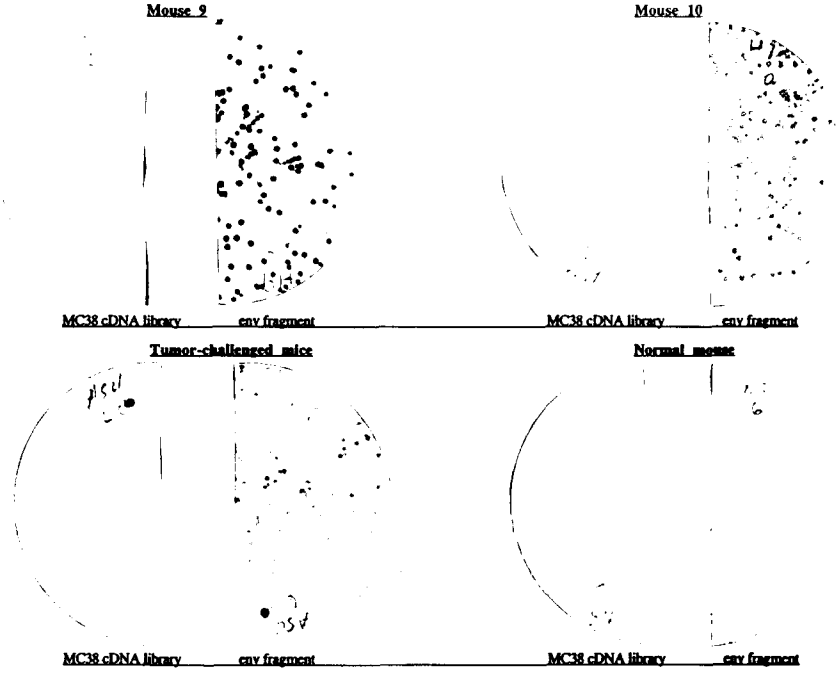


Figure 1. Assay of humoral immune response subsequent to polynucleotide immunization with a cDNA encoding the murine endogenous retroviral envelope protein. Ten mice were immunized as described below. Shown are reactive plaques visualized using sera from two animals immunized by direct DNA injection ("Mouse #9", "Mouse #10"); pooled sera from mice immunized with whole, irradiated MC38 cells ("Tumor -challenged mice", positive control); or serum from a naive mouse ("Normal mouse", negative control). Serum from each mouse was incubated with the MC38 cDNA library (left), as a negative control for nonspecific binding, or purified plaques encoding a portion of the envelope protein (env fragment, right side of each filter pair). Sera from mice immunized with the 1.3 kb fragment of the *env* gene by DNA injection are strongly reactive with envelope protein.

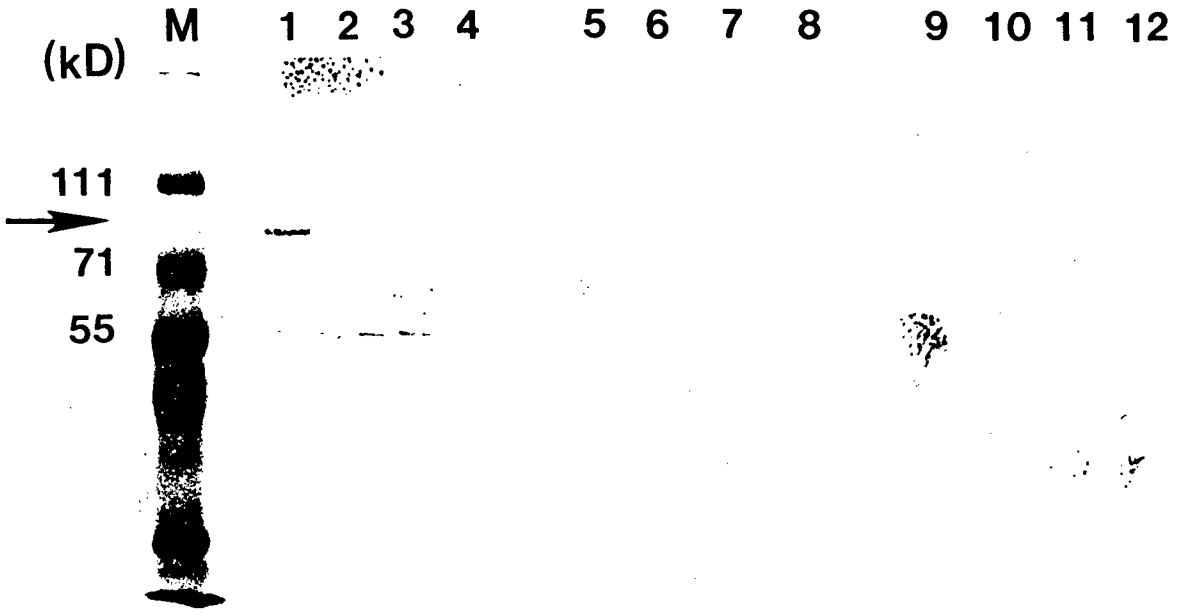
Methods: Plaques encoding the envelope gene fragment or the library (negative) were induced to express protein by IPTG-soaked filters. Filters were lifted, incubated with sera (preabsorbed with E. coli phage lysate and diluted 1:250), washed, incubated with an alkaline phosphatase conjugated goat-anti-mouse IgG and visualized by a color reaction. DNA immunization was accomplished by intramuscular injection of 6 x 50 µg of plasmid DNA encoding a 1.3 kb fragment of the env gene driven by a CMV promoter.

Thus, this serological method of screening for tumor antigens has resulted in the identification of an endogenous retroviral protein as a tumor antigen in MC38 cells. This proviral sequence appears to be activated and expressed in this colon adenocarcinoma cell line. Our characterization has defined this as a nonmutant antigen which may act as a shared tumor antigen in mice. Of note, this finding has led us to hypothesize that human endogenous retroviral proteins may act as tumor antigens in breast cancer. We are pursuing studies to test this hypothesis in collaboration with another investigator at this institution (Dr. Feng Wang-Johanning). To this end, we have performed PCR analysis on breast cancer cell lines and tumor tissues and have found evidence of expression of some endogenous retroviral elements. Current studies are focused on using in situ hybridization to more accurately compare expression of these sequence in tumor and matched normal tissue.

A second putative tumor antigen derived from MC38 cells has been undergone preliminary characterization. The 3.0 kb clone identified in the initial screen was sequenced and found to represent a portion of the mouse homologue of the human ATRX gene - a zinc finger helicase with transcriptional regulatory properties (accession #AF026032). This gene is known to regulate expression of several target genes, including α globin, perhaps by an effect on chromatin conformation (8) Mutation of this gene and loss of function is associated with a mental retardation syndrome in humans. This transcript was found to be expressed at high levels in B16 melanoma cells, with moderate expression in MC38 and CT26 colonic carcinoma cells and low expression in 4T07, 4T1, and normal colonic epithelium (not shown). In an initial evaluation of immune response, polynucleotide immunization using the 3 kb clone resulted in induction of humoral immune response in all immunized animals. We have sequenced approximately 80% of the clone identified from MC38 cells and have not yet found any point mutations compared to the normal sequence.

B. SEREX analysis of a cDNA expression library derived from human breast cancer cell lines has identified two candidate breast tumor associated antigens.

Having validated the SEREX approach in an animal model, we next applied this strategy to the identification of human breast tumor antigens. These studies utilized a cDNA library (kindly provided by J.M. Ruppert at UAB) constructed from four breast cancer cell lines: MCF-7, MDA-MB453, T47D, and ZR75-1. Immunoscreening of this library with sera from six individuals with advanced breast cancer has led to the identification of two candidate tumor antigens. (*Stmt of Work, Aim 1, Task 1, months 1-18*) One clone, designated 7-3, is reactive with sera from 7 of the 14 breast cancer patients. In contrast, the clone is nonreactive with serum from 10 normal individuals. The reactive antibodies are IgG in origin, however, we have not yet defined the subtype. Partial sequence analysis of this clone (*Stmt of Work, Aim 2, Task1, months 6-24*) revealed it to be the apparent human homologue of the recently described mouse *Mem3* gene (9) . This gene is reported to be expressed at high levels in embryos and at low levels in adult tissues. Its exact function is unknown, but it shares homology with a yeast sorting protein. By Northern analysis (*Stmt of work, Aim 2, Task 2, months 10-30*) we detected expression of a transcript approximately 2.3 kb in size in all of the cell lines used to construct the cDNA library, with the highest level of expression in MDA-MB-453 cells. Expression was also high in the ovarian cancer cell line, SKOV3. Expression was also detected in cultured normal breast epithelial cells. Northern blot analysis will now be performed on blots which contain RNA from breast tumor and matching normal tissue to determine if there is differential expression in these tissues. To determine the size of the protein expressed in our clone, we also performed Western blot analysis of the protein encoded by clone 7-3 using patient sera for detection. These studies demonstrated the presence of a reactive protein of approximately 80 kDa in size, similar to the predicted molecular weight of Mem3. As expected, the protein was reactive with sera from breast cancer patients, but not healthy individuals.



Lanes: 1-4 Serum: Breast Cancer Patient #4
1. Protein: Phage lysate encoding 7-3 + IPTG
2. Protein: Phage lysate encoding 7-3, no IPTG
3. Protein: Phage lysate - no insert + IPTG (negative control)
4. Protein: Phage lysate - irrelevant insert (negative control)

Lanes: 5-8 Serum: Normal Individual
5. Protein: Phage lysate encoding 7-3 + IPTG
6. Protein: Phage lysate encoding 7-3, no IPTG
7. Protein: Phage lysate - no insert + IPTG (neg)
8. Protein: Phage lysate - irrelevant insert (neg)

Figure 2. Western blot analysis of phage lysates encoding the human homologue of Mem-3 (hMem-3). E coli phage lysates were prepared, electrophoresed and transferred to a PVDF membrane. Membranes were incubated with patient serum (lanes 1-4) or serum from a normal individual (lanes 5-8). A protein of the predicted size (~80 kDa) was detected using serum from a breast cancer patient (Lane 1).

Methods: Approximately 50 µg of total protein from phage lysates were subjected to electrophoresis and transferred to a PVDF membrane. Sera was preabsorbed with E. coli phage lysate, diluted to 1:250 and incubated for one hour with the membrane. The filter was then washed, incubated with a goat anti-human IgG, and binding was detected by a colorimetric assay.

To further extend this analysis, we are currently preparing recombinant protein from this clone in a prokaryotic system (6-His tag, Qiagen). Our goal is to produce this protein in sufficient quantity and quality to develop an ELISA-based screen to test sera from cancer patients and healthy individuals. Such an assay will allow us to rapidly screen a large number of samples and determine the frequency of antibodies directed against this protein in different populations.

We have also identified a second candidate breast tumor antigen from this cDNA library through the SEREX method. Partial sequence analysis this clone (2-1-1) has revealed no strong homology with sequences reported in the sequence databases. This protein was reactive with two of six breast cancer sera. Current studies are focused on delineating the open reading frame of this 3.0 kb insert. Partial sequence analysis has identified an open reading frame of at least 106 amino acids. Analysis of phage lysates has revealed the presence of a protein approximately 40 kD in size produced by phage with the

3.0 kb insert. We are currently exploring expression of this gene in normal and breast tumor tissue by Northern blot analysis.

C. SEREX analysis of a cDNA expression library derived from a human tumor and screened with autologous sera has identified one additional putative tumor antigen. Finally, we have constructed a cDNA library from a human breast tumor and screened with autologous serum. A large primary tumor was obtained from a patient with advanced breast cancer and mRNA isolated. A cDNA library was constructed and screened using the patient's serum. This analysis has resulted in the identification of a reactive protein which shows homology to a reported expressed sequence tag (EST) present in an ovarian cancer cDNA library (accession #AA074820). This antigen is reactive in 2 of 20 breast cancer patients, and was not reactive with serum from 10 normal individuals nor 7 individuals with head and neck carcinomas. Both breast cancer patients with reactivity had exclusive IgG_1 antibody response to this antigen. Northern blot analysis has demonstrated the expression of this gene in both normal and tumor tissue.

DISCUSSION

We have validated serological screening of cDNA expression libraries as a relatively facile method of identifying tumor antigens in a mouse model of adenocarcinoma, and extended these studies to humman breast cancer. With regard to the statement of work, we have constructed one breast tumor cDNA library and screened it with autologous sera. Over a million plaques from this library were screened with autologous sera. We have screened a second cDNA library (derived from breast cancer cell lines) with autologous sera from six different patients. We used pooled samples (2 samples per pool) to screen more than a million plaques per patient. These combined screenings have resulted in the identification of three potential tumor associated antigens. Thus we have made significant progress with regard to task 1, aim 1. We intend to continue screening our cDNA libraries with additional patient sera as it becomes available. We would also like to construct at least one additional cDNA library over the course of the upcoming year to be screened with autologous and allogeneic sera.

As delineated in the statement of work outlined for specific aim #2, we have performed an initial molecular analysis of those putative tumor antigens identified in specific aim #1. This has included partial sequence analysis and comparison with databanks to identify known genes, initial investigation of expression pattern, analysis of reactivity in breast cancer patients and other cancer patients.

CONCLUSIONS

We have demonstrated that in a mouse model of adenocarcinoma, SEREX analysis offers a comparatively rapid means of identifying immunogenic tumor proteins. In the mouse system we identified two tumor antigens and characterized them at the molecular level. One of these antigens, the envelope protein of the endogenous ecotropic retrovirus has been shown to be a tumor antigen in other model systems. We demonstrated that in the MC38 syngeneic model, the expressed sequence is not mutated with respect to the germline sequence. Thus this protein is immunogenic based on its expression in tumor tissue and lack of expression in normal adult tissue. We also showed that this protein is likely to be a shared tumor antigen in several models of mouse tumors. This observation has led us to explore the possibility of human endogenous retroviral proteins acting as tumor antigens in breast cancer; and preliminary studies to test this hypothesis have been initiated. We have also identified a nuclear protein involved in regulation of gene expression as a potential antigen in the mouse model. Further studies are needed to elucidate the role of this protein in tumor cells.

We have extended our animal studies to the human and have thus far succeeded in identifying three potential breast tumor antigens. Our work will now focus on more completely characterizing these genes with respect to sequence variation and expression pattern. We will also perform more extensive analysis of the immune response elicited by these antigens and the relevance of that immune response to breast cancer. These studies will allow us to determine if these proteins may be useful for diagnositic testing, staging and/or for immunotherapy of this disease.

REFERENCES

1. Schlichtholz, B., Legros, Y., D. Gillet, C. Gillard, M. Marty, D. Lane, F. Calvo, T. Soussi. The immune response to p53 in breast cancer patients is directed against immunodominant epitopes unrelated to the mutational hot spot. Cancer Res. 52: 6380-6384, 1992.

2. Lubin, R., B. Schlichtholz, D. Bengoufa, G. Zalcman, J. Tredaniel, A. Hirsch, C. Caron de Fromentel, C. Preudhamme, P. Fenaux, G. Fournier, P. Mangin, P. Laurent-Puig, G. Pelletier, M. Schlumberger, F. Desgrandchamps, A. Le Duc, J.P. Peyrat, N. Janin, B. Bressac, and T. Soussi. Analysis of p53 antibodies in patient with various cancers define B-cell epitopes of human p53: distribution on primary structure and exposure on protein surface. Cancer Res. 53:5872-5876, 1993.

3. Disis, M.L., E. Calenoff, G. McLaughlin, A.E. Murphy, W. Chen, B. Groner, M. Jeschke. N. Lydon, E. McGlynn, R.B. Livingston, R. Moe, and M.A. Cheever. Existent T-cell and antibody immunity to HER-2/neu protein in patients with breast cancer. Cancer Res. 54:16-20, 1994.

4. Sahin, U., O. Tureci, H. Schmitt, B. Cochlovius, T. Johnnes, F.S. Schmits, G. Luo, I, Schobert, M Pfreundschuh. Human neoplams elicit multiple specific immune responses in the autologous host. Proc. Natl Acad Sci. 92:11810-11813, 1995.

5. Tureci, O., U. Sahin, I. Schobert, M. Koslowski, H.. Schmitt, H-J Schild, F. Stenner, G. Seitz, H-G Rammensee, M. Preundschuh. The SSX-2 gene, which is involved in the t(X;18) translocation of synovial sarcomas, codes for the human tumor antigen HOM-MEL-40. Cancer Res 56:4766-4772, 1996.

6. Gure A.O., N.K. Altorki, E Stockert., J.M. Scanlan , L.J. Old, and Y-T Chen . Human lung cancer antigens recognized by autologous antibodies: Definition of a novel cDNA derived from the tumor suppressor gene locus on chromosome 3p21.3. Cancer Res. 58:103401041, 1998.

7. Herr, W. Nucleotide sequence of AKV murine leukemia virus. J. Virol. 49: 471-478, 1984.

8. Picketts, D.J., A.O. Tastan, D.R. Higgs, R.J. Gibbons. Comparison of the human and murine ATRX gene identifies highly conserved, functionally important domains. Mamm. Genome 9:400-403, 1998.

9. Hwang, S.Y., L.E. Benjamen, B. Oh, J.L. Rothenstein, S.L. Ackerman, R.S. Beddington, D. Solter, B.B. Knowles. Genetic mapping and embryonic expression of a novel, maternally transcribed gene, Mem3. Mamm. Genome 7:586-590, 1996.

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Serological Identification of the Murine Endogenous Leukemia Proviral Envelope Protein as a Tumor Antigen in MC38 Cells

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ABSTRACT

Cancer immunotherapy strategies would benefit from a more complete understanding of the spectrum of antigens capable of eliciting anti-tumor immune responses. Although several tumor antigens have been identified based on their ability to activate cytotoxic T cells (CTLs), this approach is not readily accomplished for many tumor types. An alternative strategy based on humoral immune responses has been developed, termed serological identification of antigens by recombinant expression cloning (SEREX). We applied SEREX to the identification of tumor antigens in the murine colon adenocarcinoma cell line, MC38. Immunization of syngeneic C57BL/6 mice with MC38 induced a protective immune response with concomitant production of anti-MC38 antibodies. Screening of an MC38-derived expression library with immune sera resulted in the identification of the endogenous ecotropic leukemia virus envelope protein (*env*) as a tumor antigen. This sequence is one of the many endogenous retroviral sequences present in the normal mouse genome. Northern blot analysis demonstrated high levels of expression of the *env* transcript in MC38 cells and several other murine tumor cell lines, while expression in normal colonic epithelium was absent. Southern blot analysis suggested that amplification of the *env* gene has occurred in MC38 cells. Analysis of the expressed *env* sequence indicated that the encoded protein is not mutated as compared to the normal genomic sequence. It thus represents a nonmutated, overexpressed tumor antigen.

Key Words: tumor antigen, endogenous retrovirus, antibody, immunotherapy, adenocarcinoma

INTRODUCTION

The concept of cancer immunotherapy is predicated on the existence of tumor-associated antigens (TAAs) against which the host is capable of mounting an immune response leading to tumor rejection. To this end, immune responses in tumor-bearing individuals have been described for a variety of mutant tumor antigens such as mutant p53 (1-3) and ras (4-7), as well as nonmutant tumor proteins including erbB-2 (8, 9), MART-1 (10, 11), MAGE-1 (12, 13), tyrosinase (14, 15), and MUC-1 (16). Such tumor-specific responses suggest that the immune system may be amenable to strategies that further induce or enhance patients' immune responses to their own tumor cells. To date, however, only a limited number of tumor-associated antigens have been identified. The identification and characterization of additional TAAs have relevance for better understanding of the interaction of cancer and the immune system as well as for the development of effective cancer vaccines. Similarly, the molecular identification of murine tumor antigens is important for the development of animal models to investigate the feasibility of immunotherapeutic approaches.

Several methods have been employed to isolate and clone TAAs. Due to the critical role of cytolytic T cells (CTLs) in mediating antitumor immunity, these approaches have largely consisted of the isolation of antigens recognized by CTLs which have been expanded *in vitro*. Tumor antigens have been identified in this manner by biochemical means through acid elution of antigenic peptides bound to major histocompatibility complex class I molecules (17). A second, genetic, approach assays CTL responsiveness to target cells transfected with cDNA libraries to identify immunogenic peptides (18, 19). While these approaches have proven valuable for TAA identification in some tumor types, particularly melanoma, widespread application is hampered by difficulties in establishing long-term CTL cultures. In addition, it has not yet been established that CTLs generated *in vitro* are representative of the tumor-specific CTLs *in vivo*. Thus, complementary approaches for the identification of novel immunogenic tumor antigens are warranted.

The activation of CTL responses is dependent on T-helper cells and is usually accompanied by the induction of humoral immunity. Indeed, humoral immune responses to known TAAs have been described in tumor-bearing individuals (1, 2, 20-24). The presence of antibodies directed towards known tumor antigens suggests its use for the identification of novel TAAs, and defines the antigens as immunogenic. The feasibility of such a strategy for identifying tumor antigens was demonstrated recently by Pfreundschuh and coworkers, who identified both known and novel tumor antigens using autologous sera from cancer patients to screen tumor-derived expression libraries (25), an approach they termed "serological identification of antigens by recombinant expression cloning" (SEREX). This technique has succeeded in expanding the scope of human tumor antigens (26). By comparison, the catalogue of cloned mouse tumor antigens remains small.

Mouse models of syngeneic tumors provide a useful system in which to investigate the nature of TAAs. In this regard, the MC38 colonic adenocarcinoma cell line was derived from C57BL/6 mice following subcutaneous injection of the carcinogen 1,2-dimethylhydrazine (27). These cells reliably produce tumors when injected subcutaneously in syngenic mice. Importantly, immunization of naive syngeneic mice with MC38 cells induces an immune response which mediates tumor rejection upon challenge with an otherwise lethal dose of live cells. Thus, these cells express cryptic tumor antigens which stimulate antitumor immune responses *in vivo*. Here we describe the application of SEREX technology towards the identification of antigens in MC38 cells. This approach identified the endogenous ecotropic retroviral envelope protein as a tumor antigen in these cells.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

MC38 and MC38-CEA-2 (28) cells were kindly provided by Dr. Steven Rosenberg at the National Cancer Institute and were maintained in Dulbecco's modified Eagle's' medium (DMEM) supplemented with 10% fetal calf serum (FCS). B16 is a mouse melanoma cell line which arose spontaneously in C57BL/6 mice (29). These cells were maintained in Minimal Essential Medium-Eagle's (EMEM) with 10% FCS. The mouse lymphoma cell line EL4 (30), a chemically induced line derived from a C57BL/6 mouse, was maintained in DMEM supplemented with 10% horse serum. The mouse mammary tumor cell lines 4T01 and 4T07 are sublines of a spontaneously arising mammary tumor in a BALB/cfC$_3$H mouse (31), and were maintained in DMEM/F12 supplemented with 10% FCS. CT26 cells are colonic adenocarcinoma cells from a BALB/c mouse induced by N-nitroso-N-methylurethane (32), and they were cultured in RPMI 1640 with 10% FCS. P815, a mastocytoma line derived from a DBA/2 mouse (33), was maintained in RPMI 1640 with 10% FCS; and C2C12, a mouse myoblast cell line (ATCC), was maintained in DMEM with 10% FCS.

Immunization Protocol

To elicit anti-MC38 immune responses, groups of twelve mice were immunized in one of three ways. Group I mice were injected with 5 x 10^5 MC38 cells into the right foot pad and tumors were excised 10-14 days later. Ten of twelve mice survived the amputation and were available for challenge on day 29 with 3x10^5 cells injected subcutaneously into the flank. Eight of the ten mice rejected the day 29 tumor challenge and received a second identical tumor challenge on day 79, followed 14 days later by exsanguination to harvest sera. Group II mice were injected subcutaneously on days 1 and 15 with 1 x 10^6 MC38 cells which had been irradiated with 15,000 rads. Mice were then challenged with 3 x 10^5 non-irradiated tumor cells on day 29. Three animals developed tumors and were sacrificed, while the remaining 9 animals were challenged with an additional 3 x 10^5 non-irradiated tumor cells on day 54. Sera were collected from these animals 14 days later. Group III animals were immunized with a plasmid

DNA encoding human carcinoembryonic antigen (CEA) and challenged with MC38 cells stably transfected to express CEA (MC38-CEA-2, 28). We have previously shown that polynucleotide immunization with a CEA-encoding plasmid results in reliable rejection of MC38-CEA-2 cells (34). Thus, 12 mice received intramuscular injections of 50 μg of plasmid encoding CEA on days 1 and 15 followed by tumor challenge with 3 x 10^5 MC38-CEA-2 cells on days 29, 41, and 54. Eight of twelve mice successfully rejected all three challenges with MC38-CEA-2 cells, and sera were collected from these mice 14 days after the last challenge. In each of the three immunization experiments described above, tumors grew in twelve of twelve naive control mice, validating each challenge with MC38 or MC38-CEA-2 tumor cells.

Assay for Antibody Response to Intact Tumor Cells

To determine whether the various immunization strategies had elicited an antibody response to MC38 tumor cells, a cell binding assay was performed (35). Tumor cells were washed with phosphate buffered saline (PBS) and 5x10^5 cells were aliquoted per tube in 100 μl of PBE (PBS with 1% bovine serum albumin). Each tube then received 100 μl of a 1:10 dilution of immune sera or normal mouse sera in PBE, and the cell/serum mixture was incubated at room temperature for one hour on an orbital shaker. Cells were then washed with 4 mls of PBS to remove unbound antibody. After washing, the cells were resuspended in 100 μl of PBS containing 0.1% pigskin gelatin and 100 ng of ^{125}I-labeled Staphylococcus aureus protein A (approximately 200,000 cpm per tube), and incubated for one hour at room temperature with shaking. Staphylococcus protein A (SPA) was obtained from Sigma Chemical Company and was radiolabeled by the Iodogen method, Pierce Chemical Company. The cells were washed with 4 mls of PBS and counted in a gamma scintillation counter. The specific activity of the ^{125}I-labeled SPA was used to convert cpm bound to number of molecules bound per cell as previously described (35). The SPA binding to cells incubated in PBE only (no serum) was subtracted as non-specific binding. MC38-CEA-2 cells incubated with CEA-specific monoclonal antibody (COL-1) served as a positive control.

cDNA Library Construction and Immunoscreening

Total RNA from MC38 cells or MC38-CEA-2 cells was isolated using RNA Stat 60 RNA isolation reagent (Tel-Test B), and mRNA was isolated on oligo-dT beads (PolyA-Quik mRNA Isolation Kit, Stratagene). A cDNA library was constructed in the Zap Express vector (Stratagene), which is capable of directing expression in both prokaryotic and eukaryotic hosts. Briefly, mRNA was reverse transcribed using an oligo dT primer with an internal *Xho*I site as well as a random primers which included a *Xho*I restriction site at the end. The reverse transcription reaction included 5-methyl dCTP. After second strand synthesis, *Eco*RI adapters were added by ligation and the cDNA was restricted with *Xho*I. cDNA fragments were cloned into the directional λZapExpress vector, packaged and used to infect *E.coli* cells. As a preliminary characterization of the library, inserts from twenty random plaques were amplified by the polymerase chain reaction (PCR) and demonstrated insert sizes ranging from approximately 300 bp to 3.5 kb with greater than 90% recombinants (not shown).

For immunoscreening, recombinant plaques were plated at a density of approximately 20,000 plaques per 150 mm plate, and protein expression was induced using nitrocellulose filters saturated with isopropyl b-D-thioglactoside (IPTG). Filters were lifted, blocked with 1% BSA in Tris-buffered saline (TBS; 20 mM Tris, pH 7.5, 150 mM NaCl) and screened with sera from mice immunized as above to detect reactive plaques. Primary mouse sera were preabsorped with *E. coli* phage lysate (Stratagene) and diluted 1:250 for screening. After incubating filters with diluted mouse sera, the filters were washed with TBST (TBS with 0.05% Tween 20 [Sigma]) and incubated with alkaline phosphate-conjugated goat anti-mouse IgG antibodies (Stratagene) at a dilution of 1:5,000 for one hour at room temperature. After washing, an NBT/BCIP colorimetric assay was used to identify positive clones. Positive plaques were purified to clonality for further study. Isotype analysis was performed using subtype specific antibodies (Clonotyping System, Southern Biotechnology Associates), according to the manufacturer's directions.

DNA Sequencing

Phagemid DNA from positive plaques was rescued by *in vivo* excision using a helper phage system (Stratagene). Purified DNA was sequenced by dideoxy sequencing using Sequenase 2.0 and ^{35}-S labeled dATP (USB). Approximately 250 basepairs of sequence was determined at both the 5' and 3' ends using T3 and T7 primers, respectively. Sequences were searched against the National Center for Biotechnology (NCBI) databases using the BLASTN program.

To sequence the envelope gene expressed in MC38 for comparison with C57BL/6 genomic sequences, PCR products were generated from reverse transcribed MC38 total RNA and C57BL/6 genomic DNA. The 2.6 kb coding region was amplified using the following primers:

5' GAGAAAAGCTTCGCCCCCGATAAACCATGGAG 3'

5' GCGCAAGCTTTGCACCAGCAAAAGGCTTTATT 3'

*Hin*dIII sites are underlined. Sequencing of the PCR products was performed using an IBI automated sequencer with the PCR primers as well as internal sequencing primers.

Northern Blot Analysis

Total RNA was isolated from cultured cells using RNA Stat-60 (Tel-Test B) according to the manufacturer's directions. To isolate RNA from colonic epithelium, a section of colon was isolated, opened longitudinally, and the epithelium was recovered by gentle scraping. Epithelial cells were homogenized in RNA Stat-60 and processed as for the cultured cells. Approximately eight micrograms of total RNA from each sample were electrophoresed on a 1% denaturing agarose gel and transferred to a nylon membrane (Amersham). The blot was prehybridized for 2 hours at 65°C in a solution of 6xSSC, 5x Denhardt's, 100µg/ml sheared salmon sperm DNA, 1 mM EDTA and 0.5% SDS. Two hundred ng of a random-primed ^{32}-PdCTP labeled probe was generated corresponding to bp 6004 - 6543 of the *env* gene, a *Bgl*II to *Bam*HI fragment. This probe recognizes only the ecotropic envelope sequence (36, 37). The probe was hybridized overnight in the same solution at a concentration of 20 ng/ml. After hybridization, the blot was washed successively in 2xSSC with 0.5 % SDS, 2x SSC with 0.1% SDS, and 0.1x SSC with 0.1% SDS; and the blot was autoradiographed at -70°C for a period of 1 to 4 days.

To control for loading differences, the blot was stripped with boiling 0.1% SDS and rehybridized with a probe specific for the mouse beta-actin gene (Stratagene).

Southern Blot Analysis

For Southern blot analysis, genomic DNA from MC38 cells, B16 cells, and normal colonic epithelial cells was isolated by lysis in 50 mM Tris, pH8, 10 mM EDTA, 10 mM NaCl, 1% SDS and treatment with 100μg/ml Proteinase K overnight at 50°C. Following phenol/chloroform extraction, the DNA was precipitated with ethanol and resuspended in 10mM Tris, 1 mM EDTA, pH 8.0. Approximately 10 μg of DNA was subjected to digestion with the appropriate restriction enzyme, electrophoresed on a 1% agarose gel and transferred to a nylon membrane (Amersham). The membrane was hybridized with an *env*-specific probe as above.

RESULTS

Immunization and Detection of Antibodies Directed Against MC38 Antigens

The overall experimental design is represented in Fig. 1. Three groups of mice were immunized to generate sera reactive with MC38 cells. Group I mice were injected with MC38 cells with subsequent tumor excision, and group II animals were immunized with irradiated MC38 cells. Both of these groups were then challenged with live MC38 cells, which were rejected by most animals (17 of 22). A third group of animals was immunized with plasmid DNA encoding human CEA (pCEA) and challenged with MC38 cells expressing CEA, as previously reported (34). Eight of twelve animals in this group rejected three consecutive tumor challenges. After the immunization schedules were completed, the presence of MC38-reactive antibodies was detected in an MC38 cell binding assay (Fig. 2). All three groups of immunized mice demonstrated high levels of antibody binding to intact MC38 cells, whereas naive mouse serum did not. Mice immunized with pCEA and challenged with MC38-CEA-2 cells produced antibodies to the parental MC38 cells in addition to CEA-specific antibodies, as manifested by increased antibody binding to MC38-CEA-2 cells compared to parental MC38 cells. Thus, these serum samples contained antibodies useful for detection of cryptic tumor associated antigens in MC38 cells.

Identification of Reactive Plaques

Sera from immunized animals were used to screen MC38-derived cDNA libraries. Reactive plaques were then purified to homogeneity in secondary and tertiary screens. Screening of approximately 10^6 plaques resulted in the identification of two independent positive plaques. Amplification of the insert of one of these plaques using the polymerase chain reaction demonstrated the presence of an insert approximately 3.0 kb in size. Partial sequence analysis of this insert revealed the presence of an open reading frame, and further characterization of this clone is underway. The second positive plaque had an insert size of approximately 1.3 kb. Sera from animals immunized with MC38 cells by all three routes described above were strongly reactive with the 1.3 kb plaque (Fig. 3A). Isotype analysis revealed that reactive antibodies were predominantly IgG_{2a} and IgG_{2b}, with IgM and IgG_1 being weakly reactive.

Dideoxy sequencing was performed on rescued plasmid DNA using the T3 and T7 primers present in the vector. A search of the NCBI database revealed identity of the clone with a portion of the endogenous ecotropic leukemia proviral sequence (MuLV) including a 3' portion of the envelope sequence as well as the 3'LTR (Genbank accession numbers J01998 and U63133) (Fig. 3B). This clone includes basepairs 7071 to 8374 of the published sequence (38), encoding the amino acids 430 to the C-terminus of the envelope protein. The encoded sequence includes approximately the last one-third of the gp70 portion of the protein as well as the entire transmembrane domain. A 99 bp deletion of the 3' LTR was also noted, as discussed below.

Assessment of *env* mRNA Expression in Murine Tumor Cells

Northern blot analysis was performed to determine if the *env* gene is actively transcribed in MC38 cells (Fig. 4). Total RNA was isolated from MC38 cells and several other murine tumor cell lines. These included the C57BL/6 derived cell lines B16 (melanoma) and EL4 (lymphoma), as well as the BALB/c-derived colonic line, CT26, and the mammary tumor cell lines 4T1 and 4T07. The mastocytoma cell line P815 from DBA/2 mice was also analyzed, as was the nontransformed myoblast cell line C2C12. The endogenous ecotropic *env* gene has been previously implicated as a melanoma antigen in B16 cells (39), and more recently was identified as the origin of an immunodominant major histocompatibility complex class I restricted antigenic peptide in CT26 cells (40). Total RNA was also isolated from the normal colonic epithelium of a C57BL/6 mouse. High levels of *env* gene expression were detected in MC38 cells, B16, CT26 and 4T07. Expression was also noted in 4T1 and EL4, with very low levels of expression in P815 cells. Expression of the proviral sequences was not detected in the myoblast line C2C12 as well as in normal colonic epithelium. Thus the ecotropic endogenous retrovirus is expressed in a variety of murine tumor cell lines, including both spontaneously arising (B16, 4T1, and 4T07) and carcinogen-induced tumors (MC38, EL-4, P815, and CT26) .

Analysis of the *env* Gene by Southern Blot

We next investigated the genomic structure of the proviral sequence in MC38 cells. Endogenous retroviruses may become activated as the result of a point mutation and remain single copy (41), or activation may be accompanied by amplification or rearrangement of the proviral sequence (42). C57BL/6 cells normally contain a single copy of the endogenous ecotropic proviral sequence, which resides on chromosome 8 (43). To determine if this MuLV proviral sequence was amplified and/or rearranged upon activation in MC38 cells, Southern blot analysis was performed. Genomic DNA from normal colon, MC38 cells and B16 cells was digested with the restriction enzymes *Bam*HI, *Hind*III, *Pst*I, *Pvu*II, and *Kpn*I . Hybridization of the DNA with the ecotropic-specific *env* probe revealed the presence of novel fragments in the MC38 DNA, not found in the normal DNA or in B16 DNA (Fig. 5). This was evident when restriction enzymes cutting once within the proviral sequence and once in the flanking DNA were used, suggesting that the MC38 proviral sequence has likely undergone amplification and reintegration into new genomic sites.

Analysis of the *env* sequence

To determine if the envelope sequence expressed in MC38 cells represented a mutant or nonmutant tumor antigen, we next sequenced the expressed *env* sequence and compared it to the AKV published sequence (38) and to the endogenous sequence in C57BL/6 genomic DNA. The MC38 *env* sequences were amplified from total RNA by polymerase chain reaction after reverse transcription. The normal sequence was amplified from genomic DNA extracted a C57BL/6 mouse. Previously reported coding sequence differences between B16 *env* (also derived from a C57BL/6 mouse) and that of the AKV (38) were either not found in the MC38 expressed sequence (nucleotides 6116, 6419, 7017 and 7085; reference 38), or were found to be the same in the MC38-derived *env* and the C57BL/6 genomic sequence. Thus the differences between the MC38 and AKV-derived sequences are likely to represent variations between the mouse strains. Also of note, as reported by others (39, 42), the *env* gene expressed in MC38 cells has a 99 bp deletion in the 3'LTR compared with AKV; however, this change also appears to be present in the C57BL/6 genomic sequences when size is examined by PCR (data not shown).

DISCUSSION

A limited number of murine tumor antigens have been described to date, and the ability of these antigens to elicit effective antitumor immunity is currently an area of active investigation. While prospects for the development of immunotherapeutic modalities have recently been improved by advances in the field of immunology (44, 45), knowledge of the spectrum of tumor antigens important in mediating immune interactions is needed. Additionally, it is important to catalogue the nature of murine tumor antigens, such that a comparison with human cancers can be explored and more appropriate animal models can be developed for evaluating immunotherapeutic approaches to cancer. We have used a strategy for tumor antigen identification which exploits the humoral immune responses that accompany induction of antitumor immunity, and identified the MuLV endogenous ecotropic *env* gene-encoded protein as a tumor antigen in MC38 colon adenocarcinoma cells.

Endogenous retroviruses are relatively stable elements present in multiple copies throughout the murine genome and inherited in a Mendelian fashion (46). C57BL/6 mice carry a single copy of an endogenous ecotropic virus, and it is defective and transcriptionally silent in normal tissues (39). Activation of endogenous retroviruses spontaneously or following exposure to carcinogens has been described in several mouse strains. In DBA/2 mice, activation of an endogenous MuLV was accomplished by a point mutation which restores a myristilation site in the *gag* gene (41). In other cases, activation has occurred as a result of transcomplementation by, or recombination with, other endogenous retroviral sequences (47-49), or by amplification and rearrangement of the locus (42). Here activation is associated with appearance of new restriction sites. The appearance of several new bands suggests that amplification and reintegration events are likely to have occurred, although the introduction of multiple point mutations, recombination with other endogenous retroviruses, or transcomplementation with other retroviruses may also play a role. The exact nature of the activating mutation(s) will require further study.

Although the env protein was identified in this study on the basis of humoral immunity, it has been determined to be a target of CTL activity in both C57BL/6 mice (39) and BALB/c mice (40). In fact, a peptide derived from the ecotropic MuLV env was found to be the immunodominant antigen in the CT26 cell line, and env-specific CTLs were effective in lysing tumor cells (40). The identification of a CTL-activating protein by immunoscreening further supports the use of antibody-based screening for the identification of relevant tumor antigens. Further, while most murine tumor antigens are thought to be unique to a particular tumor (50, 51), our findings suggest that *env* is a shared tumor antigen in murine cancer. This antigen is expressed in both spontaneous and mutagen-induced tumor cell lines derived from mice of different genetic background, and from a variety of tissue types. Immune responses directed towards this antigen are elicited when the animals are immunized by a variety of different means (results presented herein, 39, 40, 42). Sequence analysis revealed no apparent differences between the sequences expressed in the MC38 cells and the genomic sequences of the *env* gene, suggesting that this antigen is a true "self" antigen. Thus, env is immunogenic based on its expression in the tumor. These attributes may make this tumor antigen an ideal target for additional studies relevant to tumor vaccinology. Studies are ongoing to determine if this antigen can function as a tumor rejection antigen; i.e., can immunization with the antigen protect against tumor challenge with MC38 or other, *env*-expressing tumor cells?

The relevance of this murine tumor antigen to human tumors remains to be determined. Human endogenous retroviral elements (HERVs) are present throughout the genome. Unlike mice, however, expression of a few of these proviral sequences has been reported in some normal human tissues (52). The primary site of expression is the placenta, although low levels of expression have also been reported elsewhere including blood and salivary glands. HERV expression has also been noted in a variety of tumor cell lines. Recently, antibodies to the HERVK10 env sequence were reported to be present in a large percentage of patients with seminoma (53). These findings and the immunodominant nature of this antigen in murine models of carcinoma suggest that the role of endogenous retrovirus-encoded proteins as tumor antigens in mice and humans warrants further consideration.

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REFERENCES

1. Crawford LV, Pim DC, Bulbrook, RD. Detection of antibodies against the cellular protein p53 in sera from patients with breast cancer. Int J Cancer 1982;30:403-8.

2. Schlichtholz B, Legros Y, Gillet D, Gilliard C, Marty M, Lane D, Calvo F, Soussi T. The immune response to p53 in breast cancer patients is directed against immunodominant epitopes unrelated to the mutational hot spot. Cancer Res 1992;52:6380-4 .

3. Houbiers JG, Nijman H.W., van der Burg SH, et al. *In vitro* induction of human cytotoxic T lymphocyte responses against peptides of mutant and wild-type p53. Eur J Immunol 1993;23:2072-7.

4. Abrams SI, Hand PH, Tsang KY, Schlom J. Mutant ras epitopes as targets for cancer vaccines. Sem Oncol 1996;23: 118-34.

5. Jung S, Schleusener HJ. Human T lymphocytes recognize a peptide of single point-mutated oncogenic ras proteins. J Exp Med 1991;173: 273-6.

6. Fenton RG, Keller CJ, Hanna N, Taub DD. Induction of T-cell immunity against Ras oncoproteins by soluble protein or Ras-expressing Escherichia coli. J Natl Cancer Inst 1995;87:1853-61.

7. Abrams SI, Dobrzanski MJ, Wells DT, et al. Peptide specific activation of cytotoxic CD4+ T lymphocytes against tumor cells bearing mutated eptiopes of K-ras p21. Eur. J. Immunol 1995;2588-2597.

8. Disis M, Calenoff E, McLaughlin, G et al . Existent T-cell and antibody immunity to HER-2/neu protein in patients with breast cancer. Cancer Res 1994;54: 16-20.

9. Peoples GE, Goedegebuure PS, Smith R, Linehan DC, Yoshino I, Eberlein TJ. Breast and ovarian cancer specific cytotoxic T lymphocytes recognize the same HER2/neu-derived peptide. Proc Natl Acad Sci USA 1995;92:432-6.

10. Coulie PG, Brichard V, Van Pel A, et al. A new gene coding for a differentiation antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas. J Exp Med 1994;180:35-42.

11. Kawakami Y, Eliyahu S, Delgado CH, et al. Cloning of the gene coding for a shared human melanoma antigen recognized by autologous T cells infiltrating into tumor. Proc Natl Acad Sci USA 1994;91:3515-9.

12. van der Bruggen P, Traversari C, Chomez P, et al. A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. Science 1991;254:1643-47.

13. Toso JF, Oei C, Oshidari F, et al. MAGE-1 specific precursor cytotoxic T-lymphocytes present among tumor-infiltrating lymphocytes from a patient with breast cancer: characterization and antigen-specific activation. Cancer Res 1996;56:16-20.

14. Robbins P., el-Gamil M, Kawakami Y, Stevens E, Yannelli JR, Rosenberg SA. Recognition of tyrosinase by tumor-infiltrating lymphocytes from a patient responding to immunotherapy. Cancer Res 1994;54:3124-6.

15. Topalian SL, Rivoltini L, Mancini M, et al. Human CD4+ T cells specifically recognize a shared melanoma-associated antigen encoded by the tyrosinase gene. Proc Natl Acad Sci USA 1994;91:9461-5.

16. Ioannides CG, Fisk B, Jerome KR, Irimura T, Wharton JT, Finn OJ. Cytotoxic T cells from ovarian malignant tumors can recognize polymorphic epithelial mucin core peptides. J Immunol 1993;151:3693-03.

17. Mandelboim O, Berke G, Fridkin M, Feldman M, Eisenstein M, Eisenbach L. CTL induction by a tumour-associated antigen octapeptide derived from murine lung carcinoma. Nature 1994;369: 67-71.

18. De Plaen E, Lurquin C, Van Pel A, et al. Immunogenic (tum-) variants of mouse tumor P815: cloning of the gene of tum- antigen P91A and identification of the tum- mutation. Proc Natl Acad Sci USA 1988;85:2274-2278.

19. Malarkannan S, Serwold T, Nguyen V, Sherman LA, Shastri N. The mouse mammary tumor virus *env* gene is the source of a CD8+ T-cell-stimulating peptide presented by a major histocompatibility complex class I molecule in a murine thymoma. Proc Natl Acad Sci USA 1996;93:13991-6.

20. Disis ML, Pupa SM, Gralow JR, Dittadi R, Menard S, Cheever MA. High-titer HER-2/neu protein-specific antibody can be detected in patients with early-stage breast cancer. J Clin Oncol 1997;15:3363-7.

21. Kotera Y, Fontenot JD, Pecher G, Metzgar RS, Finn OJ. Humoral immunity against a tandem repeat epitope of human mucin MUC-1 in sera from breast, pancreatic and colon cancer patients. Cancer Res 1994;54:2856-60.

22. Mattes MJ, Thomson TM, Old LJ, Lloyd KO. A pigmentation-associated, differentiation antigen of human melanoma defined by a precipitating antibody in human serum. Int J Cancer 1983;32:717-21..

23. Ben-Mahrez K, Sorokine I, Thierry D, et al. Circulating antibodies against c-myc oncogene product in sera of colorectal cancer patients. Int J Cancer 1990;46:35-8.

24. Chinni SR, Falchetto R, Gercel-Taylor C, Shabanowitz J, Hunt DF, Taylor DD. Humoral immune responses to cathepsin D and glucose-regulated protein 78 in ovarian cancer patients. Clin Cancer Res 1997;3:1557-64.

25. Sahin U, Tureci O, Schmitt H, et al. Human neoplasms elicit multiple specific immune responses in the autologous host. Proc Natl Acad Sci USA 1995;92:11810-11813.

26. Sahin U, Tureci O, Pfruendschuh M. Serological identification of human tumor antigens. Curr Opinions Immunol 1997;9:709-16.

27. Corbett TH, Griswold DP, Roberts BJ, Peckham JC, Schabel FM. Tumor induction in the development of transplantable cancers of the colon in mice for chemotherapy assays, with a note on carcinogen structure. Cancer Res 1975;35:2434-9.

28. Robbins PF, Kantor JA, Salgaller M. Transduction and expression of the human carcinoembryonic gene in a murine carcinoma cell line. Cancer Res 1991;51:3657-3662.

29. Fidler IJ. Biological behavior of malignant melanoma cells correlated to their survival *in vivo*. Cancer Res 1975;35:218-234.

30. Herberman RB. Serological analysis of cells surface antigens of tumors induced by murine leukemia virus. J. Natl. Cancer Inst *48:* 265-271, 1972.

31. Aslakson CJ, Miller FR. Selective events in the metastatic process defined by analysis of sequential dissemination of subpopulations of mouse mammary tumor. Cancer Res 1992;52:1399-1405.

32. Griswold DP, Corbett TH. A colon tumor model for anticancer evaluation. Cancer 1975;36:2441-4.

33. Dunn TB, Potter M. A transplantable mast-cell neoplasm in the mouse. J Natl Cancer Inst 1957;18:587-95.

34. Conry RM, LoBuglio AF, Loechel F, Moore SE, Sumerel LA, Barlow DL, Curiel DT. A carcinoembryonic antigen polynucleotide vaccine has *in vivo* antitumor activity. Gene Ther 1995;2:59-65.

35. Shaw GM, Axelson J, Maglott JG, LoBuglio AF. Quantification of platelet-bound IgG by 125I-Staphylococcal protein A in immune thrombocytopenic purpura and other thrombocytopenic disorders. Blood 1984;63:154-61.

36. Chan HW, Bryan T, Moore JL, Staal SP, Rowe WP, Martin MA. Identification of ecotropic proviral sequences in inbred mouse strains with a cloned subgenomic DNA fragment. Proc Natl Acad Sci USA 1980;77:5779-83.

37. Chattopadhyay SK, Lander MR, Rands E, Lowy DR. Structure of endogenous murine leukemia virus DNA in mouse genomes. Proc Natl Acad Sci USA 1980;77:5774-8.

38. Herr W. Nucleotide seqeunce of AKV murine leukemia virus. J Virol 1984;49:471-8.

39. Hayashi H, Matsubara H, Yokota T, et al. Molecular cloning and characterization of the gene encoding mouse melanoma antigen by cDNA library transfection. J Immunol 1992;149:1223-9.

40. Huang AY, Gulden PH, Woods AS, et al. The immunodominant major histocompatibility complex class I-restricted antigen of a murine colon tumor derives from an endogenous retroviral gene product. Proc Natl Acad Sci USA 1996;93:9730-5.

41. Mercer JA, Lee KH, Nexo BA, Jenkins NA, Copeland NG. Mechanism of chemical activation of expression of the endogenous ecotropic murine leukemia provirus *Emv-3*. J Virol 1990;64: 2245-9.

42. Li M-f, Muller J, Xu F, Hearing VJ, Gorelik E. Inhibition of melanoma-associated antigen expression in B16BL6 melanoma cells transfected with major histocompatibility complex class I genes. Cancer Res 1996;56:4464-74.

43. Jenkins NA, Copeland NG, Taylor BA, Lee BK. Organization, distribution, and stability of endogenous ecotropic murine leukemia virus DNA sequences in chromosomes of *Mus musculus*. J Virol 1982;43:26-36.

44. Ridge JP, Fuchs EJ, Matzinger P. Neonatal tolerance revisited: turning on newborn T cells with dendritic cells. Science 1996;271:1723-6.

45. Shu S, Plautz GE, Krauss JC, Chang AE. Tumor immunology. JAMA. 1997;278:1972-81.

46. Coffin JM, Stoye JP, Frankel WN. Genetics of endogenous murie leukemia viruses. Ann NY Acad Sci 1989;567:39-49.

47. Bartman T, Murasko DM, Sleck TG, Turturro A, Hart R, Blank KJ. A murine leukemia virus expresssed in aged DBA/2 mice is derived by recombination of the *Emv-3* locus with another endogenous *gag* sequence. Virology 1994;203:1-7.

48. Benade LE, Ihle JN. Different serotypes of B-tropic murine leukemia viruses and association with endogenous ecotropic viral loci. Virology 1980;106: 374-86.

49. Stoye J-P, Moroni C, Coffin JM. Virological events leading to spontaneous AKR thymomas. J Virol 1991;65:1273-8 .

50. Mumberg D, Wick M, Schreiber H. Unique tumor antigens redefined as mutant tumor-specific antigens. Sem Immunol 1996;8:289-93.

51. Jaffee EM, Pardoll DM. Murine tumor antigens: is it worth the search. Curr Opin Immunol 1996;8:622-7.

52. Lower R, Lower J, Kurth R. The viruses in all of us: Characteristics and biological significance of human endogenous retrovirus sequences. Proc Natl Acad Sci USA 1996;93:5177-5184.

53. Sauter M, Roemer K, Best B, et al. Sepcificity of antibodies directed against Env protein of human endogenous retroviruses in patients with germ cell tumors. Cancer Res 1996;56:4362-5.

FIGURE LEGENDS

Figure 1. Diagrammatic representation of experimental design. Three groups of mice were immunized as described in the text and subsequently challenged with live MC38 or MC38-CEA-2 cells. Sera from immunized animals were used to screen cDNA expression libraries synthesized from MC38 mRNA.

Figure 2. Antibody response to intact tumor cells. Sera collected from mice immunized as outlined in Figure 1 were examined for antibody binding to intact tumor cells. Antisera from naive mice or immunized mice (groups I-III) were incubated with MC38 cells. In addition, sera from naive mice and animals immunized in group III were incubated with MC38-CEA-2 cells. Results are reported as the number of molecules of antibody bound per cell, as described in Methods. Values greater than twice those observed with normal mouse sera represent positive results. Binding of a CEA-specific monoclonal antibody (COL-1) to MC38 cells stably expressing human CEA (MC38-CEA-2) served as positive control.

Figure 3. Identification of a positive clone as the murine endogenous ecotropic retrovirus envelope gene. A. Reactivity of immune sera with the 1.3 kb clone. Sera from each group of immunized mice (AS1, AS2, or AS3), or from naive animals (NMS) were incubated with filters lifted from plates of purified plaques expressing 1.3 kb *env* gene (1.3 kb), or a negative control (no insert) and processed as described in methods. B. Partial sequence analysis of 1.3 kb clone aligned with the sequence of the murine endogenous ecotropic retrovirus. Approximately 200 bp of the 5' end of the 1.3 kb clone is shown in alignment with the endogenous ecotropic murine leukemia virus *env* sequence acquired from Genbank (accession numbers: J01998)

Figure 4. Northern blot analysis of *env* gene expression in normal colonic epithelium and murine tumor cell lines. A. Expression of the *env* gene was examined. Total RNA was

hybridized with an envelope-specific probe. The lower transcript presumably represents the mature, spliced *env* transcript, while the upper band represents the proviral transcript. No expression of the *env* gene was detected in normal colonic epithelium, even after prolonged autoradiography.

B. The blot was stripped and rehybridized with a mouse beta-actin probe to demonstrate approximately equal loading.

Figure 5. Southern blot analysis of the endogenous ecotropic retrovirus in C57BL/6 genomic DNA, MC38 genomic DNA, and B16 genomic DNA. A. Diagrammatic representation of restriction sites in the ecotropic endogenous proviral sequence. The location of the ecotropic-specific probe is indicated. B. Genomic DNA from C57BL/6 mouse colon (Nrl), MC38 cells and B16 cells was digested with the indicated restriction enzymes and hybridized with the ecotropic specific *env* probe. The arrow indicates the location of the endogenous proviral sequence in normal C57BL/6 genomic DNA.

Figure 1

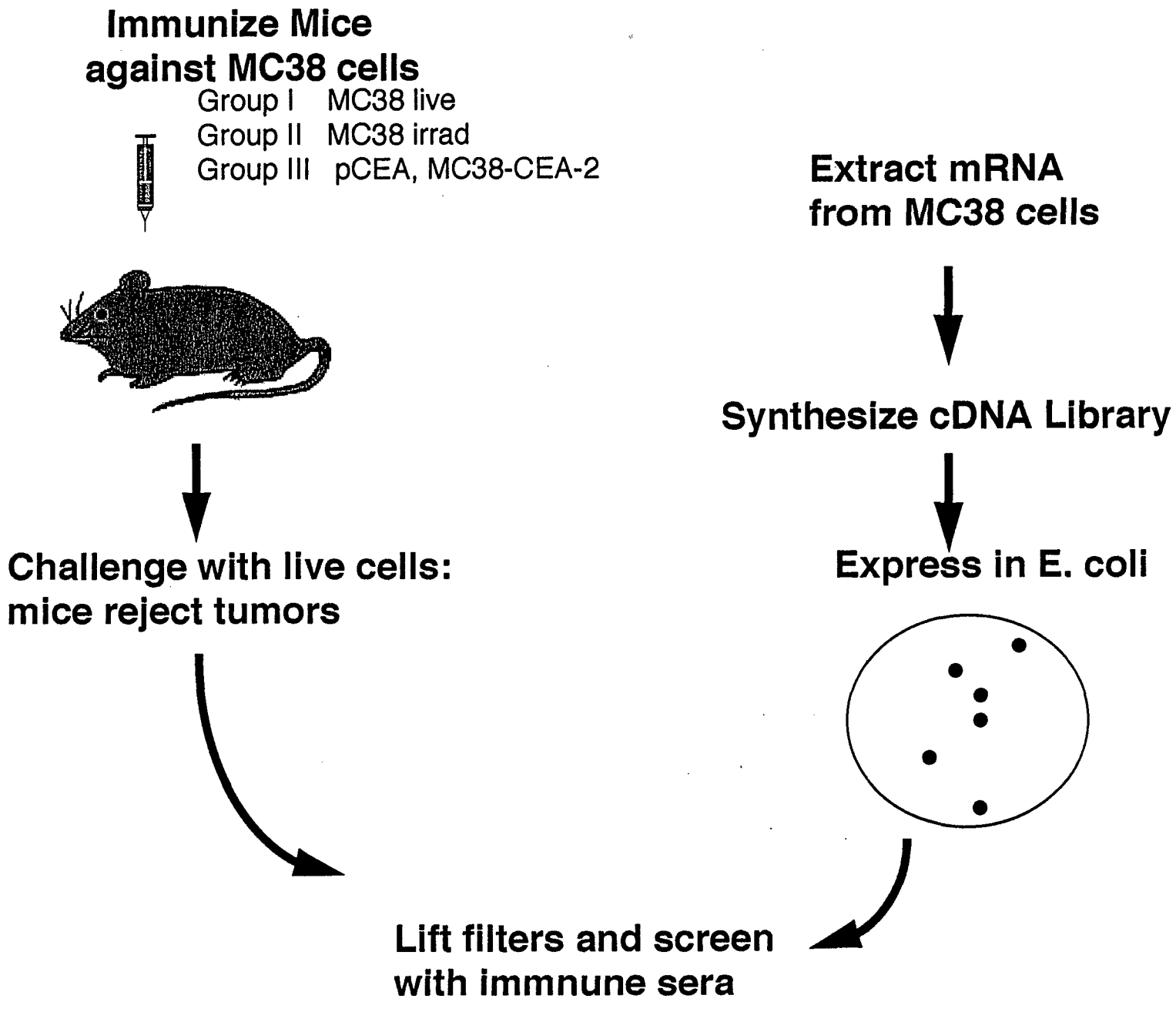
Immunize Mice
against MC38 cells
Group I MC38 live
Group II MC38 irrad
Group III pCEA, MC38-CEA-2
Challenge with live cells:
mice reject tumors
Extract mRNA
from MC38 cells
Synthesize cDNA Library
Express in E. coli
Lift filters and screen
with immnune sera

Figure 2

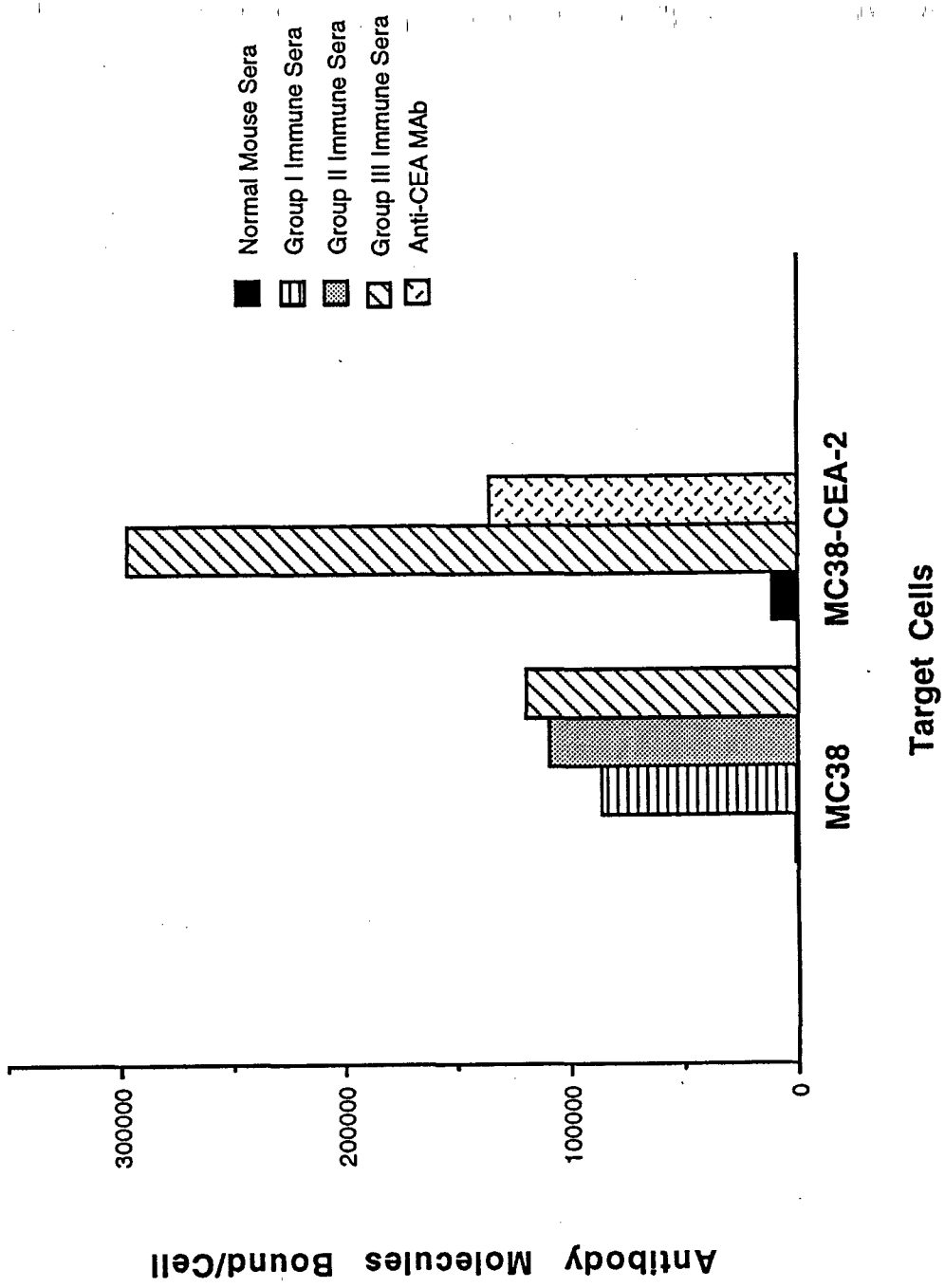
Normal Mouse Sera
Group I Immune Sera
Group II Immune Sera
Group III Immune Sera
Anti-CEA MAb
Antibody Molecules Bound/Cell
300000
200000
100000
0
MC38
MC38-CEA-2
Target Cells

Figure 3A

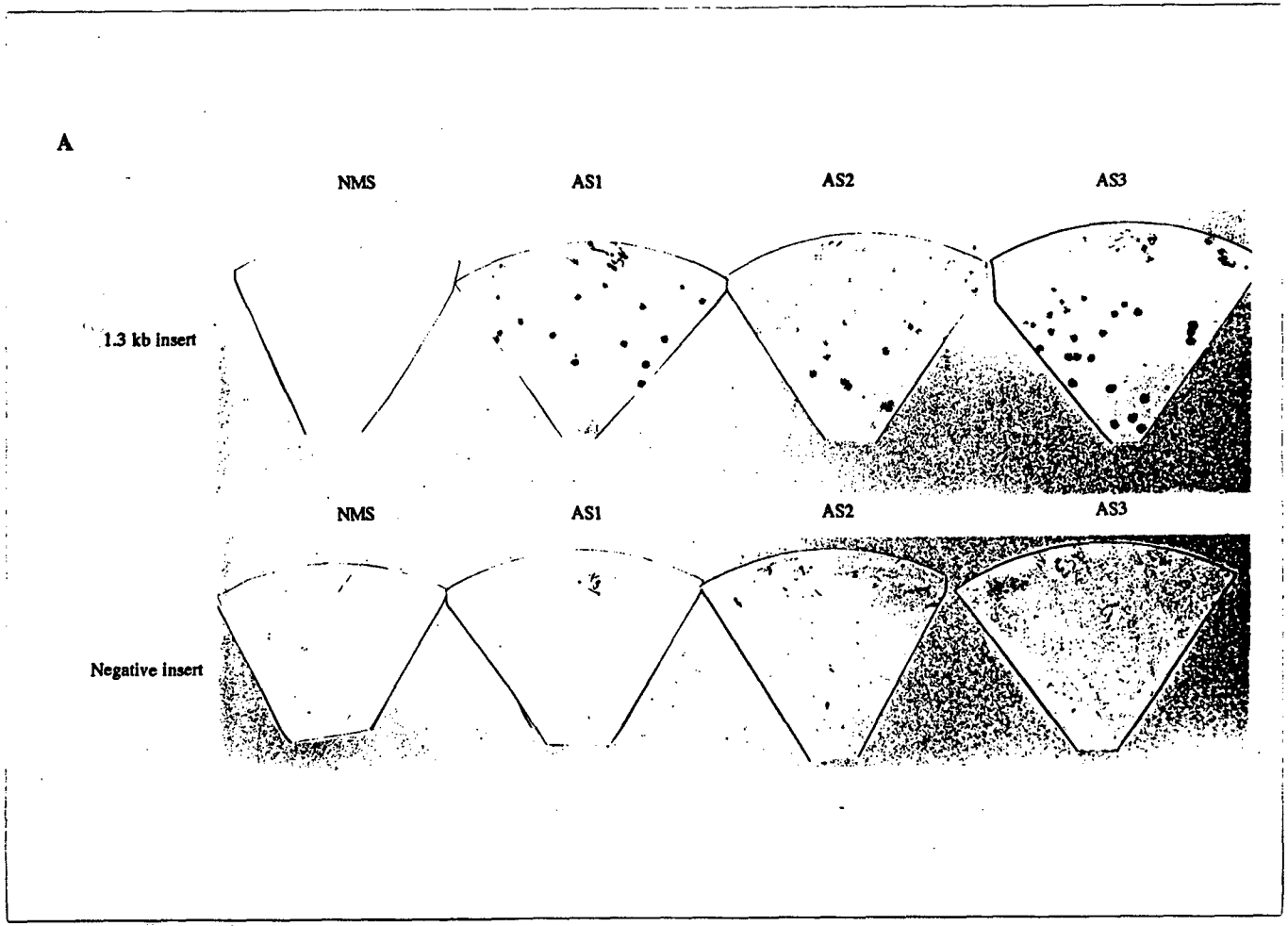

Figure 3B

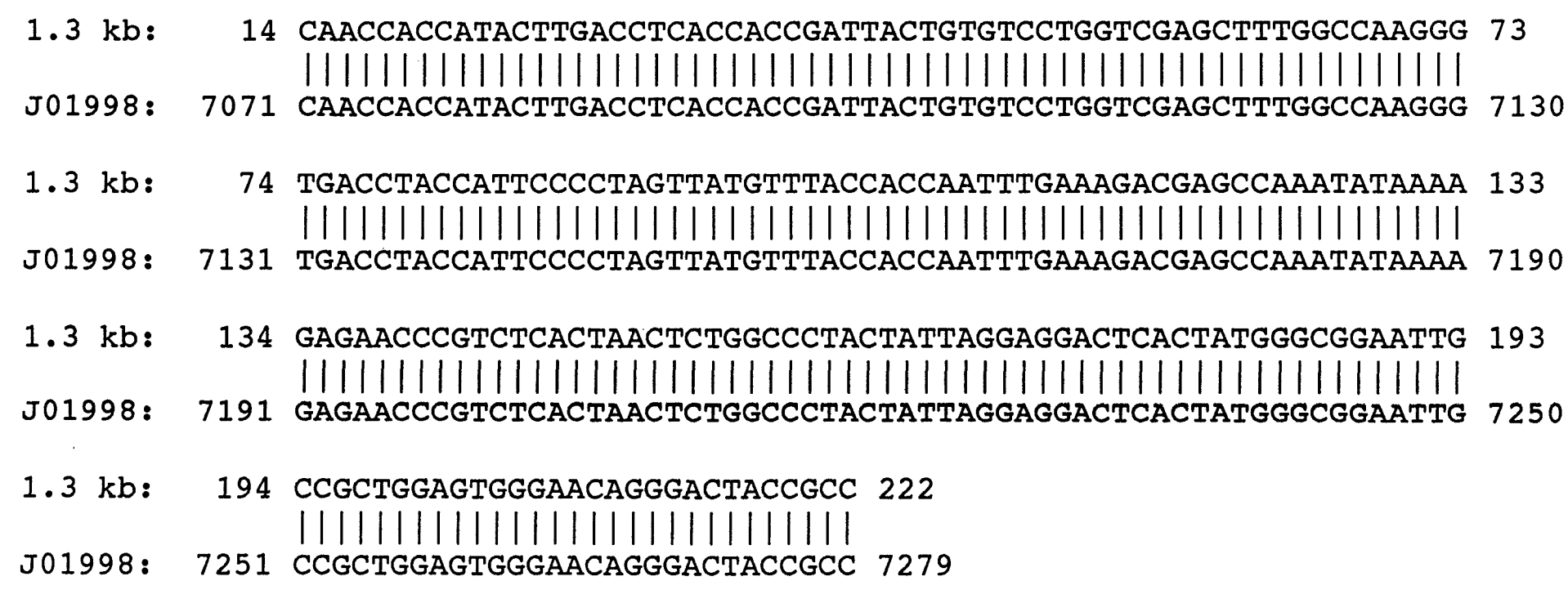
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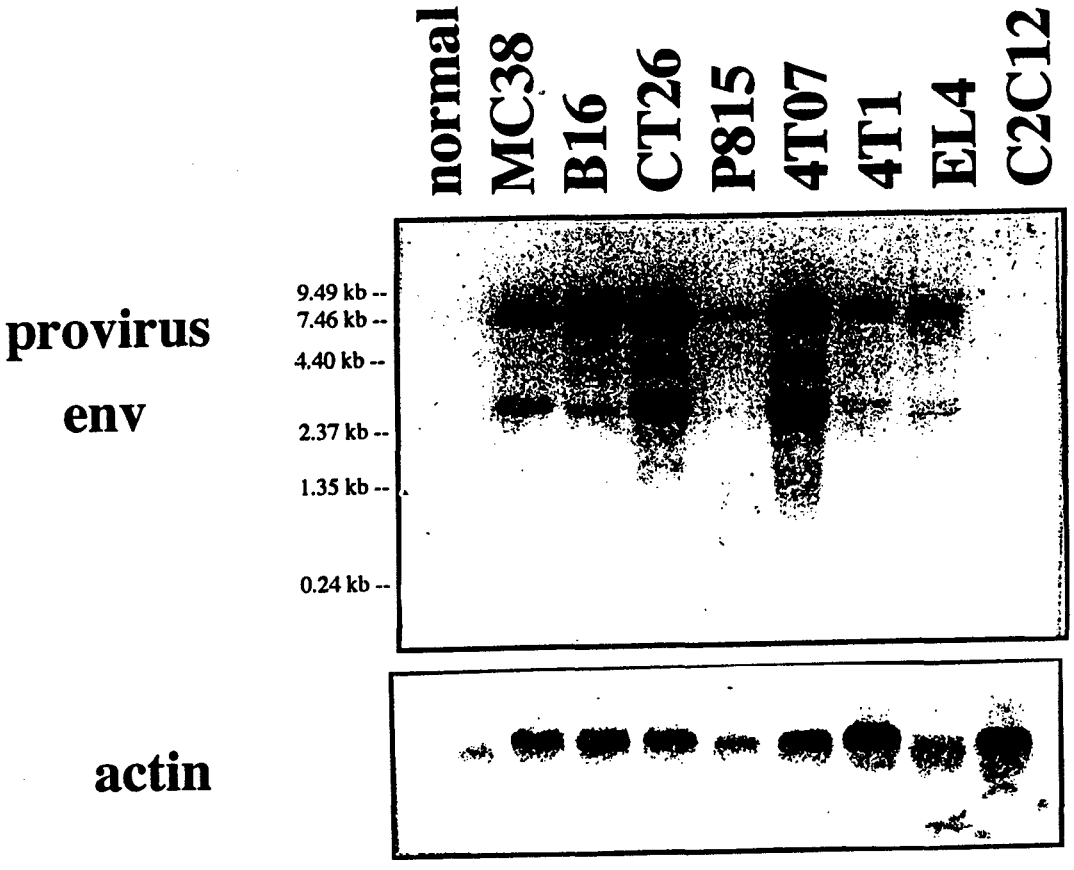
Figure 4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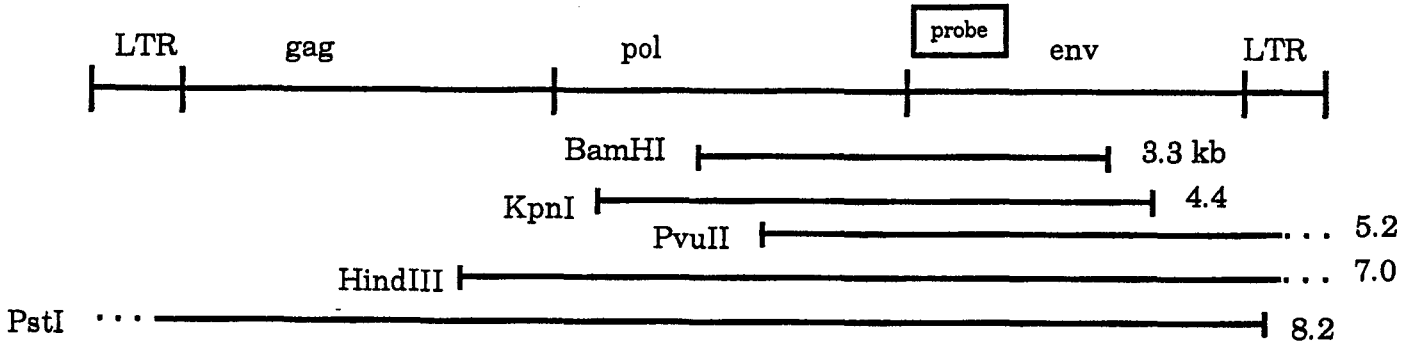
actin

Figure 5A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pol
probe
env
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BamHI
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HindIII
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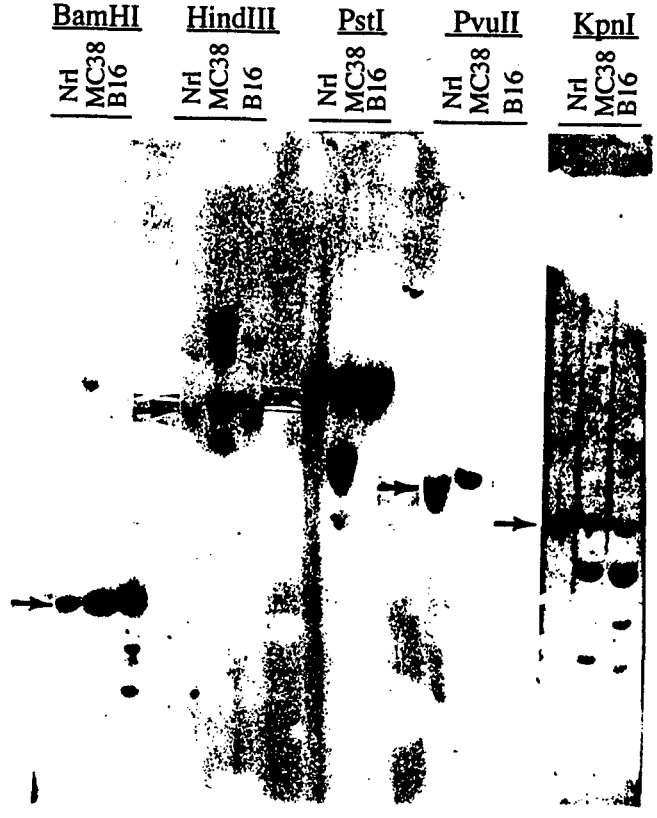
PstI
8.2

Figure 5B



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